

**TITLE PAGE**

**Characterisation of healthy donor-derived T-cell responses specific to telaprevir diastereomers**

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**Running title: The immunological basis of drug hypersensitivity**

## Abstract

Telaprevir, a protease inhibitor, was used alongside PEGylated interferon- $\alpha$  and ribavirin to treat hepatitis C viral infections. The triple regimen proved successful; however, the appearance of severe skin reactions alongside competition from newer drugs restricted its use. Skin reactions presented with a delayed onset indicative of a T-cell mediated reaction. Thus, the aim of this study was to investigate whether telaprevir and/or its diastereomer, which is generated in humans, activates T-cells. Telaprevir in its S-configured therapeutic form and the R-diastereomer were cultured directly with PBMC from healthy donors prior to the generation of T-cell clones by serial dilution. Drug-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell clones responsive to telaprevir and the R-diastereomer were generated and characterised in terms of phenotype and function. The clones proliferated in a dose-dependent manner and secreted IFN- $\gamma$ , IL-13, and granzyme B in response to culture with telaprevir and the diastereomer at the same concentrations. In contrast, the telaprevir M11 metabolite did not stimulate T-cells. The CD8<sup>+</sup> T-cell response was MHC I-restricted and dependent on the presence of soluble drug. Flow cytometric analysis showed that clones expressed chemokine receptors CCR4 (skin homing) and CXCR3 (migration to peripheral tissue) and one of three distinct TCR V $\beta$ s; TCR V $\beta$  2, 5.1, or 22. These data show the propensity of both R- and S-forms of telaprevir to generate skin-homing cytotoxic T-cells that may induce the adverse reactions observed in human patients.

**Key words:** drug hypersensitivity, human, immune system

## Introduction

Hepatitis C (HCV) is a serious, potentially life-threatening viral infection that affects an estimated 71 million individuals worldwide (WHO, 2017). Although up to a quarter of infected patients effectively clear the virus (WHO, 2017), the vast majority develop chronic HCV infection. The associated inflammation ultimately leads to severe liver disease, including hepatocellular carcinoma, liver fibrosis and cirrhosis, due to which hepatitis C is the most common indication for liver transplantation in the US (Verna and Brown, 2006). HCV has been traditionally treated with a dual regimen of PEGylated IFN- $\alpha$  and ribavirin which provide a sustained antiviral response ( $<10$  IU/ml) in just 39% of patients. In contrast, an updated triple treatment regimen including telaprevir (TVR, VX-950), increases the frequency of patients that achieve viral control to 70% (Lang, 2007). Telaprevir is an NS3/4.A protease inhibitor for use against HCV genotype 1 which prevents both the cleavage of viral proteins into active polypeptides for viral assembly, and the deactivation of hepatic cellular proteins essential for mediating the interferon cascade and mounting a viral response (Jesudian *et al.*, 2012; Morikawa *et al.*, 2011; Smith *et al.*, 2011). While administered orally as a single S-configured diastereomer, telaprevir spontaneously forms the corresponding R-diastereomer (figure 1a, b), which is approximately 30-fold less pharmacologically active (Garg *et al.*, 2012).

Despite enhanced viral suppression, the triple regimen is associated with an increased risk of adverse cutaneous reactions, with triple telaprevir-containing therapy causing a severe rash in 4.8% of patients compared to just 0.4% with the standard dual therapy. Of more concern, a small subset of patients treated with telaprevir develop life-threatening cutaneous drug hypersensitivity reactions including drug rash with eosinophilia and systemic symptoms (DRESS) and Stevens Johnson syndrome (SJS) (Pavlos *et al.*, 2012; Roujeau, 2005; Roujeau *et al.*, 2013). These clinical diagnoses, alongside the lack of correlation between the severity

of telaprevir-induced cutaneous reactions and drug plasma concentration, as well as the delayed onset (median 15 days) and slow resolution (median 44 days) after drug discontinuation, are indicative of a type IV hypersensitivity reaction (Roujeau *et al.*, 2013). Such delayed drug hypersensitivity reactions are thought to be mediated by the activation and subsequent cytotoxic action of drug-specific T-cells, which have been previously isolated from patients with hypersensitivity to a diverse array of drugs (Kim *et al.*, 2015; Lichtenfels *et al.*, 2014; Meng *et al.*, 2017; Usui *et al.*, 2017). Despite a reported correlation between the level of the T-cell-derived cytotoxic mediator granulysin and the severity of telaprevir-induced skin reactions (Suda *et al.*, 2015), telaprevir-specific T-cells have not been identified. Furthermore, no specific HLA alleles are associated with telaprevir-induced skin reactions of any severity (Roujeau *et al.*, 2013).

To circumnavigate the inability of animal models to predict hypersensitivity, *in vitro* models that utilise T-cells from healthy human donors have been developed that are successful at generating and characterising drug-specific T-cells (Bell *et al.*, 2013; Gibson *et al.*, 2017; Monshi *et al.*, 2013; Sullivan *et al.*, 2018). Critically, these assays enable the modulation of reported susceptibility factors and the identification of the antigen, whether parent compound or metabolic derivative, responsible for the initial, highly-regulated activation of T-cells. Telaprevir undergoes extensive hepatic metabolism and forms a range of metabolites, including M11, which was identified as potentially immunogenic due to a positive read out for skin sensitizing potential in a guinea pig maximisation test. (FDA application Number 201917; Garg *et al.*, 2012) (figure 1c). In order to provide an understanding of telaprevir immunogenicity, we utilised *in vitro* peripheral blood mononuclear cell (PBMC) drug bulk cultures to assess the propensity for telaprevir-derived antigens to activate T-cells isolated from drug-naïve healthy human donors.

## Materials and methods

**Isolation of PBMC from drug-naïve healthy human donors:** Venous blood samples (120ml) were taken from seven telaprevir-naïve healthy human donors who had provided informed written consent as directed by the Liverpool local research ethics committee. A density gradient separation technique was performed to isolate the PBMC population from whole venous blood using lymphoprep (Axis-shield, Dundee).

**PBMC bulk culture:** PBMCs ( $1 \times 10^6$ /well; 48-well plate; 660ul total) were cultured for 14 days with either the S- or R-diastereomer of telaprevir (5-20uM), or the M11 metabolite (20uM). Cultures were fed with R9 medium (RPMI 1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 25 µg/ml transferrin, 10% human AB serum [Innovative Research], 25 mM HEPES buffer, 2 mM L-glutamine) supplemented with IL-2 on days 6 and 9. On day 14, cultures for the same antigen but of differing concentrations were harvested and pooled. A sample of PBMCs were frozen for later use at  $10\text{--}20 \times 10^6$  cells/ml at 1:1 ratio of R9 medium to 80% human AB serum, 20% DMSO (total volume, 1 ml). Cryovials were stored at  $-80^\circ\text{C}$  for 24-48 hrs before transfer to  $-150^\circ\text{C}$  for longer term storage. Any remaining PBMCs were used for functional studies. Briefly,  $1 \times 10^5$ /well antigen-exposed PBMCs were re-exposed in duplicate wells (96-well plate, 200ul total) to either the S- or R-diastereomer of telaprevir for 48 hours ( $37^\circ\text{C}/5\% \text{CO}_2$ ). Antigen re-exposed cultures were then pulsed with [ $^3\text{H}$ ] thymidine (0.5 µCi/well) and subject to a further 16 hr incubation before analysis of incorporated radioactivity as measure of drug-specific proliferation using a Microbeta Trilux 1450 LSC beta counter (PerkinElmer, Cambridge, U.K.).

**Serial dilution and T-cell cloning:** T-cell clones were generated from PBMC bulk cultures using serial dilution and mitogen-driven expansion (Mauri-Hellweg *et al.*, 1995). Briefly, cells were plated at 1 cell/well (96well U-bottomed plate) in a restimulation cocktail ( $5 \times 10^4$  irradiated allogeneic PBMC/well, 10ul/ml PHA, 5ul/ml IL-2) and cultured for 14 days ( $37^\circ\text{C}/5\% \text{ CO}_2$ ). Cultures were fed on day 5 and then every two days subsequently with R9 medium supplemented with IL-2. Additionally, autologous EBV-transformed B-cells (EBV) were generated from PBMC to function as an immortalised antigen presenting cell line.

To probe for antigen-specificity, expanded T-cell clones ( $5 \times 10^4$ /well; 96 well plate; total volume, 200  $\mu\text{l}$ ) were cultured ( $37^\circ\text{C}/5\% \text{ CO}_2$ ) in duplicate per experimental condition with irradiated autologous EBVs ( $1 \times 10^4$ /well)  $\pm$  the S- or R-diastereomer of telaprevir (10  $\mu\text{M}$ ) or the M11 metabolite (20uM). After 48 h, [ $^3\text{H}$ ] thymidine was added before a further 16 h culture prior to analysis of cellular proliferation by scintillation counting. T cell clones with a stimulation index (mean cpm drug-treated wells / mean cpm of control wells) of  $> 1.5$  were repetitively stimulated with allogeneic PBMCs ( $5 \times 10^4$ /well; 96 well plate; total volume, 200  $\mu\text{l}$ ) in R9 medium supplemented with PHA (5  $\mu\text{g}/\text{ml}$ ) and IL-2 for further expansion.

**T-cell clone characterisation assays:** Those clones that responded to telaprevir-derived antigens in a second confirmatory proliferation assay were further expanded and characterised. To define cross-reactivity between telaprevir diastereomers and the M11 metabolite, T-cell clones ( $5 \times 10^4$ /well; 96 well plate; total volume, 200  $\mu\text{l}$ ) were cultured ( $37^\circ\text{C}/5\% \text{ CO}_2$ ) in triplicate with autologous irradiated EBVs ( $1 \times 10^4$ /well) and either diastereomer (5-20uM) or M11 (20uM) for 48 hours prior to proliferative analysis as described above. In order to explore the requirement for antigen uptake and processing, EBVs were pulsed with telaprevir for 1-16

hrs. After the allotted exposure period, drug-exposed EBVs were washed in PBS and used to restimulate cells as above in the absence of soluble drug. Alternatively, to determine whether antigen was presented in the context of MHC, EBVs were first pre-cultured with either MHC class I or II blocking antibodies or their corresponding isotype controls (5ul; BD Biosciences, Oxford, UK) for 30mins. MHC blocked EBVs were then washed and included in the proliferation assay.

ELISpot was used to characterise the drug-specific release of specific cytokines and cytolytic molecules from T-cell clones. The release of IFN- $\gamma$ , IL-13, IL-22, and granzyme B in response to telaprevir-derived antigens was visualised by the ELISpot procedure provided by the manufacturer (Mabtech, Nacka Strand, Sweden). Flow cytometry was utilised to characterise T-cell clone phenotype, including clone CD4<sup>+</sup> or CD8<sup>+</sup> coreceptor expression, to assess the expression profile for a defined chemokine receptor panel (CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CCR9, CCR10, E-cadherin, CLA, CXCR3, CXCR6), and to determine TCR V $\beta$  protein expression using the IOTest Beta Mark TCR V $\beta$  repertoire kit (Immunotech, Beckman Coulter, UK). Briefly, aliquots of T-cells were stained with fluorescence-conjugated antibodies before incubation on ice in the dark for 20 min. Cells were then washed with PBS and resuspended in 200ul 10% FBS/PBS prior to data acquisition (minimum 5x10<sup>4</sup> events) using a FACS CANTO II flow cytometer. Data was analysed using FACS DIVA or Cyflogic software (CyFlo Ltd., Finland).

## Results

**Weak telaprevir-specific proliferative response from healthy donor-derived T-cell cultures:** PBMC bulk cultures established with either the S- or R-diastereomer of telaprevir were restimulated every 2-3 weeks to promote further expansion of antigen-specific T-cells. Cultures with high cell recovery were tested for antigen specificity before being subject to serial dilution for T-cell cloning. While responses to the model drug immunogen nitroso sulfamethoxazole (SMX-NO) were clearly detectable from PBMC bulk cultures in all donors (figure 2), the majority of cultures exposed to telaprevir diastereomers failed to proliferate (figure 2i, 2ii; representative donors 1-3). However, T-cells from donor 2 responded weakly in response to the R-diastereomer.

**Identification of CD4<sup>+</sup> and CD8<sup>+</sup> telaprevir antigen-specific T-cells:** To explore whether telaprevir-specific T-cells are present below the limit of detection in the PBMC assay, T-cell cloning was performed on cells derived from all 7 telaprevir-exposed cultures. Initial testing identified the drug-specific proliferation ( $SI > 1.5$ ) of T-cell clones from 3/7 donors (figure 3). After expansion, a further triplicate proliferation culture confirmed the presence of telaprevir-responsive T-cell clones. Five and thirty five T-cell clones derived from PBMC cultures containing the S- and R-diastereomer, respectively remained drug-responsive during repetitive mitogen-driven expansions and were used for the mechanistic studies described below.

Irrespective of the antigen used for initial culture, all clones proliferated in the presence of either diastereomer to a similar extent at similar concentrations (figure 4). Flow cytometry determined that all telaprevir diastereomer-responsive T-cell clones from donor 3 were CD8<sup>+</sup>



T-cells, while a mixed phenotype was observed from the 13 suitable for analysis from donor 2, with 10 CD4<sup>+</sup> (76.9%) and 3 CD8<sup>+</sup> (23.1%) T-cell clones identified.

In stark contrast, cloning performed on PBMC bulk cultures with the M11 metabolite from 3 healthy donors failed to identify M11-responsive T-cell clones.

### **Telaprevir-responsive T-cells secrete cytotoxic and pro-inflammatory mediators:**

ELISpot was utilized to probe for the telaprevir-induced secretion of cytokines and cytolytic molecules from the CD8<sup>+</sup> T-cell clones. All clones secreted IFN- $\gamma$  to a similar degree upon exposure to either telaprevir diastereomer (figure 5 - average spot count across 7 clones shown: IFN- $\gamma$ ; medium,  $81 \pm 47.9$ ; 10 $\mu$ M S-diastereomer,  $282 \pm 77.4$ ; 10 $\mu$ M R-diastereomer,  $288 \pm 53$ ). Drug-induced secretion of IL-13 and the cytotoxic mediator granzyme B was similarly observed. In stark comparison, none of the aforementioned mediators were secreted when telaprevir-responsive T-cell clones were exposed to the M11 metabolite (figure 6a). The lack of cross-reactivity was further confirmed by a negative proliferative response to the M11 metabolite in these clones (figure 6b). Secretion of IL-22 was not detected in response to telaprevir diastereomers, despite its reported involvement in inflammatory skin conditions (figures 5 & 6).

### **Telaprevir-induced T-cell responses are MHC-restricted and occur independent of antigen presenting cell processing:**

To assess the presentation of telaprevir to T-cells, telaprevir-specific T-cell clones were first cultured with telaprevir-pulsed autologous irradiated EBV-transformed B-cells free of soluble drug. While T-cells strongly proliferated in response to soluble drug, they were not stimulated by drug-pulsed antigen presenting cells at either time

point (1 or 16 hrs; figure 7a). Further investigation using HLA blocking antibodies focussed on the requirement for HLA in the activation of T-cells. The telaprevir-induced CD8<sup>+</sup> T-cell proliferative response was diminished by blocking HLA class I molecules (figure 7b), but not the corresponding isotype control. These data infer that telaprevir is directly presented on HLA class I molecules to passing CD8<sup>+</sup> T-cells, without a need for antigen uptake and processing.

**Telaprevir responsive T-cells express distinct TCR V $\beta$  and chemokine receptors:** To induce keratinocyte death, telaprevir-responsive T-cells must express specific homing receptors to promote migration to the skin. The expression of a diverse array of tissue-homing chemokine receptors was analysed on the drug-responsive T-cell clones, which expressed the T-cell activation marker CD69. Of the 13 migratory markers assessed, telaprevir-responsive T-cells most highly expressed CCR4 (skin homing; mean fluorescence intensity [MFI]:  $3.40 \pm 1.04$ ) and CXCR3 (migration to peripheral tissue; MFI:  $3.16 \pm 1.52$ ) (figure 8 a). Further T-cell surface expression analysis revealed a restricted pattern of TCR-V $\beta$  expression, in which there was a high expression of TCR-V $\beta$  22 (n=6, 46%), with fewer clones expressing TCR-V $\beta$  2 (n=5, 38%) and TCR-V $\beta$  5.1 (n=1, 8%). Of note, one clone expressed no identifiable TCR V $\beta$  covered by the kit, which recognises 24 specificities that account for 70% of the total repertoire (figure 8b). Thus, it is likely this T-cell clone expressed another alternative TCR-V $\beta$ .

## Discussion

As the acute stage of HCV is largely asymptomatic, the majority of patients develop a chronic infection that leads to long term, life threatening liver complications. An adaptation of the standardised dual therapy, by co-administration of telaprevir, led to > 30% increase in therapeutic response rate. However, telaprevir also enhanced the incidence of mild skin reactions, and led to a number of patients developing life threatening hypersensitivity reactions including DRESS and SJS. The appearance of new drugs with improved safety profile resulted in telaprevir being withdrawn from the market.

Drug-specific T-cells have been isolated from the blood and blister fluid of patients with other forms of severe hypersensitivity reaction, and these T-cells are thought to mediate tissue destruction. However, as yet telaprevir-responsive T-cells have not been identified or characterized in terms of phenotype and function. While samples from hypersensitive patients are informative for clinical diagnosis, they represent a memory T-cell response, and give no indication regarding the ability of the antigen to activate the more highly regulated naïve population. Thus, using PBMC drug bulk cultures we probed the immunogenicity of the telaprevir S- and R-diastereomers.

Telaprevir-responsive T-cell clones were generated from 3 out of 7 healthy volunteers; however, the frequency of identifiable drug-responsive T-cell clones was low. Although administered as the S-diastereomer, telaprevir spontaneously converts to the R-diastereomer *in vivo*. A study on Japanese HCV patients showed that the mean  $C_{\max}$  for telaprevir was 5.4  $\mu\text{M}$  at steady state (Yamada *et al.*, 2012), where maximal plasma concentrations of the R-diastereomer were almost equivalent to those of the S-diastereomer (Nakada *et al.*, 2014). In

our study, T-cell clones were derived from PBMC cultured with both the S- and R-diastereomer. In contrast to the diastereomer-specific actions of telaprevir at its pharmacological target, the hepatitis C viral enzyme NS3/4A serine protease, a high degree of T-cell cross reactivity was observed with both diastereomers. In fact, the same concentrations of S- and R-diastereomer were capable of inducing proliferate responses to a similar degree. While these data imply the parent compound is responsible for T-cell activation, the formation of a metabolite after internalisation by antigen presenting cells and subsequent presentation on HLA to passing T-cells is a possibility. One potentially immunogenic metabolite is M11, as it has previously been reported to induce a positive guinea pig maximisation test. Telaprevir-responsive T-cell clones failed to respond to M11, and moreover, cloning directly using the M11 metabolite was unsuccessful in 3 donors, including the donors who provided clones responsive towards the parent compound. While this inter-species differential response may relate to the small number of animals or humans tested between the two studies, it nonetheless stresses the importance of assessing antigenicity and immunogenicity using human models.

Telaprevir is a reversible covalent binding inhibitor of its pharmacological target. The drug interacts with a high degree of specificity with serine in position 139, which resides in the catalytic area of the protease enzyme. Dissociation of the covalently bound drug has a half-life of 58 minutes (Fowell and Nash, 2010). To investigate whether the telaprevir-responsive T-cells were activated through the formation of protein adducts by a hapten mechanism or through direct HLA binding, antigen presenting cell pulsing experiments were conducted. Haptenic drugs bind irreversibly to antigen presenting cells in the pulsing assay and stimulate T-cells after repeated washing to remove the non-covalently-bound drug. In contrast, drugs that bind directly to HLA through a reversible bond yield a negative result (Alzahrani *et al.*, 2017; Castrejon *et al.*, 2010; Schnyder *et al.*, 2000). While the telaprevir-specific T-cell response

was MHC restricted, the T-cells were only activated in the presence of soluble drug, highlighting that formation of a covalent adduct, antigen-uptake and processing by antigen presenting cells is not a requirement for T-cell activation. These data indicate that telaprevir may be able to activate T-cells expressing distinct TCR V $\beta$ s via a direct HLA binding interaction. Interestingly, telaprevir is relatively large, with a molecular weight of 680Da. This equates to the over 50% of the mass of a typical HLA class I binding peptide (assuming an average amino acid mass of 130Da and 9-10 amino acids in the peptide sequence). Thus, it will be intriguing to discover the structure of the telaprevir HLA peptide binding interaction. It seems unlikely that telaprevir will fit into a binding pocket under an HLA binding peptide. The only other possibilities are that telaprevir (1) interacts with the HLA binding peptide and projects from the HLA molecule, (2) replaces the requirement for an HLA binding peptide or (3) binds elsewhere on the HLA molecule altering the structure of the HLA binding cleft.

CD8<sup>+</sup> T-cells are designed to inflict damage and thus are the most likely mediators of keratinocyte death in patients with telaprevir hypersensitivity. Thus, CD8<sup>+</sup> cells were analysed for their cytokine secretion profile and the secretion of cytolytic molecules using ELISpot. Previously Suda *et al* reported a correlation between the level of the cytotoxic mediator granulysin and the severity of telaprevir-induced skin reactions. Moreover, they describe an early rise in serum granulysin levels with the onset of severe symptoms which fades within 6 days and therefore concluded that granulysin can be utilized as an early predictive marker for telaprevir-induced skin reactions (Suda *et al.*, 2015). In agreement with the induction of a cytotoxic response, telaprevir-responsive CD8<sup>+</sup> T-cell clones not only secreted IFN- $\gamma$  and IL-13, but also the cytotoxic mediator granzyme B in response to culture with either diastereomer. Upon release from cytotoxic T-cell granules, granzyme B enters target cells to cleave caspases and initiate apoptosis. The secretion of IL-22 was additionally monitored due to its proposed

role in inflammatory skin conditions, including psoriasis. Furthermore, IL-22-secreting cells have been identified in patients with allergic contact dermatitis and  $\beta$ -lactam hypersensitivity reactions (Akdis *et al.*, 2012; Cavani *et al.*, 2012; Eyerich *et al.*, 2010; Sullivan *et al.*, 2018). IL-22 was not secreted by telaprevir-responsive T-cells in this study.

Chemokine receptor pattern analysis on telaprevir-responsive T-cell clones detected a population with a skin-homing phenotype. While the skin homing receptor CCR10 was present, CCR4 and CXCR3 were also highly expressed. CXCR3 is predominantly expressed by Th1 T-cells while CCR4 is expressed by Th2 T-cells (Kim *et al.*, 2001). These findings, along with the antigen-specific secretion of IFN- $\gamma$  (Th1) and IL-13 (Th2), suggest the presence of a mixed Th1/2 T-cell population. CCR9, characterised as a gut homing receptor for T-cells with a role in intestinal inflammation (Agace, 2008; Bekker *et al.*, 2015), was also relatively highly expressed in comparison to other chemokine receptors. Interestingly, adverse intestinal effects with telaprevir hypersensitivity is very common ranging from diarrhoea to haemorrhoids in 25% and 12% of patients, respectively. As yet, the underlying mechanism of intestinal disruption is not defined, but this data indicates the migration of T-cells may play a role.

This study identifies and characterises the telaprevir-induced activation of T-cells from 3 out of 7 healthy drug-naïve donors that develop into skin-homing, cytotoxic T-cells, which are activated by an HLA-restricted but processing-independent mechanism. Drug-specific T-cells responded to either telaprevir diastereomer and expressed varied TCRs. Drug-responsive T-cells were induced using cultures from drug-naïve healthy donors and so define the utility of *in vitro* human platforms to explore the requirements for T-cell activation. As studies have

previously failed to associate telaprevir hypersensitivity with HLA risk alleles (Roujeau, *et al.*, 2013), the development of *in vitro* human assays to allow (a) modulation of other immune parameters and (b) inclusion of autologous keratinocytes will be key to understand the inter-individual skin-specific targeting by the immune system.

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## **List of abbreviations**

Hepatitis C, HCV; human leukocyte antigen, HLA; drug rash with eosinophilia and systemic symptoms, DRESS; Stevens Johnson syndrome, SJS; peripheral blood mononuclear cells, PBMC; mature dendritic cells, mDC; EBV-transformed B-cells, EBV; spot forming units, SFU.



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## Figure legends

**Figure 1. Chemical structures of the (A) S- (therapeutic form) and (B) R-diastereomers of telaprevir and (C) the M11 metabolite.**

**Figure 2 Healthy donor T-cell responses from PBMC bulk cultures with the telaprevir (i) S- diastereomer (ii) R-diastereomer (5-20uM), or (iii) SMX-NO (25-50uM; model drug-derived immunogen).** PBMCs ( $1 \times 10^6$ /well; 48-well plate; 660ul total) were directly cultured with antigen for 14 days prior assessment of drug specificity. [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci}$ /well) was added for the final 16 hr of the incubation and then incorporated radioactivity was measured. Data shown as proliferative stimulation index (SI; average of drug-exposed wells / average of control wells). Error bars indicate standard deviation for the average of replicate cultures.

**Figure 3. Generation of telaprevir-responsive T-cell clones.** Telaprevir diastereomer-exposed T-cells from PBMC bulk cultures were subject to serial dilution and mitogen-driven expansion. Individual T-cell clones ( $5 \times 10^4$ /well; 96 well plate; total volume, 200  $\mu\text{l}$ ) were cultured ( $37^\circ\text{C}/5\% \text{ CO}_2$ ) in duplicate per experimental condition with irradiated autologous EBVs ( $1 \times 10^4$ /well)  $\pm$  the drug antigen. After 48 h, [ $^3\text{H}$ ] thymidine was added before a further 16 h culture prior to analysis of cellular proliferation by scintillation counting. T cell clones with a stimulation index (mean cpm drug-treated wells / mean cpm of control wells) of  $> 1.5$  were selected as drug-responsive and subject to further expansion and investigation.

**Figure 4. Cross-reactivity between telaprevir-diastereomers.** Drug-responsive T-cell clones ( $5 \times 10^4$ /well; 96 well plate; total volume, 200  $\mu\text{l}$ ) generated from initial cultures with

either the R- or S-diastereomers were cultured (37°C/5% CO<sub>2</sub>) in triplicate with autologous irradiated EBV-transformed B-cells (1 x 10<sup>4</sup>/well) and either diastereomer (5-20uM) for 48 hours prior to pulsing with [<sup>3</sup>H]thymidine (0.5 µCi/well). After a further 16 hr incubation, incorporated radioactivity was counted as a measure of proliferation. Data presented as radioactive counts per minute (cpm); error bars indicate the standard deviation for the average of triplicate cultures.

**Figure 5. Cytokine and cytolytic molecule secretion from telaprevir-responsive T-cell clones.** ELISpot plates were coated with IFN-γ, IL-13, IL-22, or granzyme B capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. T-cell clones (5x10<sup>4</sup>/well; total volume, 200 µl, 96-well U-bottomed ELISpot plate) were cultured with the S- (5-10uM) or R-diastereomer of telaprevir and autologous irradiated EBV-transformed B-cells (1x10<sup>4</sup>/well). After a 48 hr incubation, the plates were washed and developed in concordance with the manufacturer's instructions. Spot forming units (SFU) counts were analysed from dry wells using an ELISpot reader.

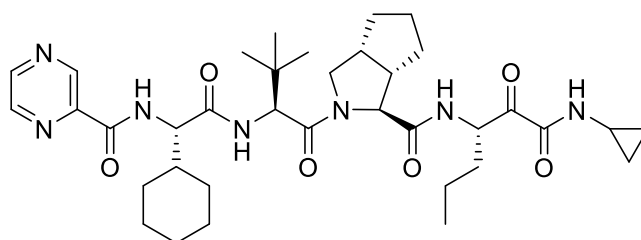
**Figure 6. Ability of the M11 metabolite to induce (A) cytokine or cytolytic molecule secretion or (B) a proliferative response in telaprevir-responsive T-cell clones.** ELISpot plates were coated with IFN-γ, IL-13, IL-22, or granzyme B capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. For both proliferation and ELISpot assays, T-cell clones (5x10<sup>4</sup>/well; total volume, 200 µl, 96-well plate) were then cultured with telaprevir (10uM) or the M11 metabolite (20uM) and autologous irradiated EBV-transformed B-cells (1x10<sup>4</sup>/well). After a 48 hr incubation, the ELISpot plates were washed and developed in concordance with the manufacturer's instructions. SFU counts were analysed

from dry wells using an ELISpot reader. Alternatively, plates for proliferation analysis were pulsed with [<sup>3</sup>H] thymidine (0.5 μCi/well) and subject to a further 16 hr incubation before measurement of incorporated radioactivity. Data presented as radioactive counts per minute (cpm), error bars indicate the standard deviation for the average of triplicate cultures.

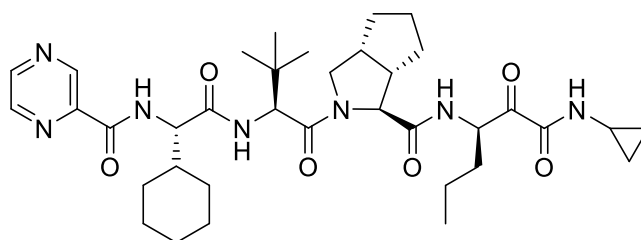
**Figure 7. Requirement for (A) antigen uptake and (B) HLA restriction for T-cell activation.** Autologous EBV were either (A) pulsed with soluble drug for 1-16 hrs prior to washing to remove free drug, or (B) cultured with HLA blocking antibodies or their respective isotype controls for 30 mins. T-cell clones (5x10<sup>4</sup>/well; total volume, 200 μl, 96-well plate) were then cultured with telaprevir (10-20uM) and pre-conditioned autologous irradiated EBV-transformed B-cells (1x10<sup>4</sup>/well). After a 48 hr incubation, cultures were pulsed with [<sup>3</sup>H] thymidine (0.5 μCi/well) and subject to a further 16 hr incubation before measurement of incorporated radioactivity. Data presented as radioactive counts per minute (cpm), error bars indicate the standard deviation for the average of triplicate cultures.

**Figure 8. TCR Vβ and chemokine profile of telaprevir-responsive T-cell clones.** T-cell clones (5x10<sup>4</sup>) were washed and stained with (A) individual Fluorochrome-conjugated antibodies specific for chemokine receptors or (B) combined Fluorochrome-conjugated antibodies specific for TCR Vβ specificities. Samples were left in the dark for 20 mins at 4°C to aid antibody binding. After which samples were washed to remove unbound antibody and fixed in 4% PFA prior to analysis using a BD FACS CANTO II flow cytometer. Data analysis was performed using cyflogic software.

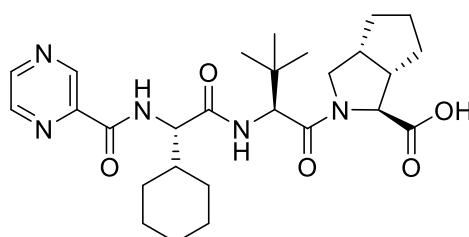
(A) Telaprevir (S-diastereomer)



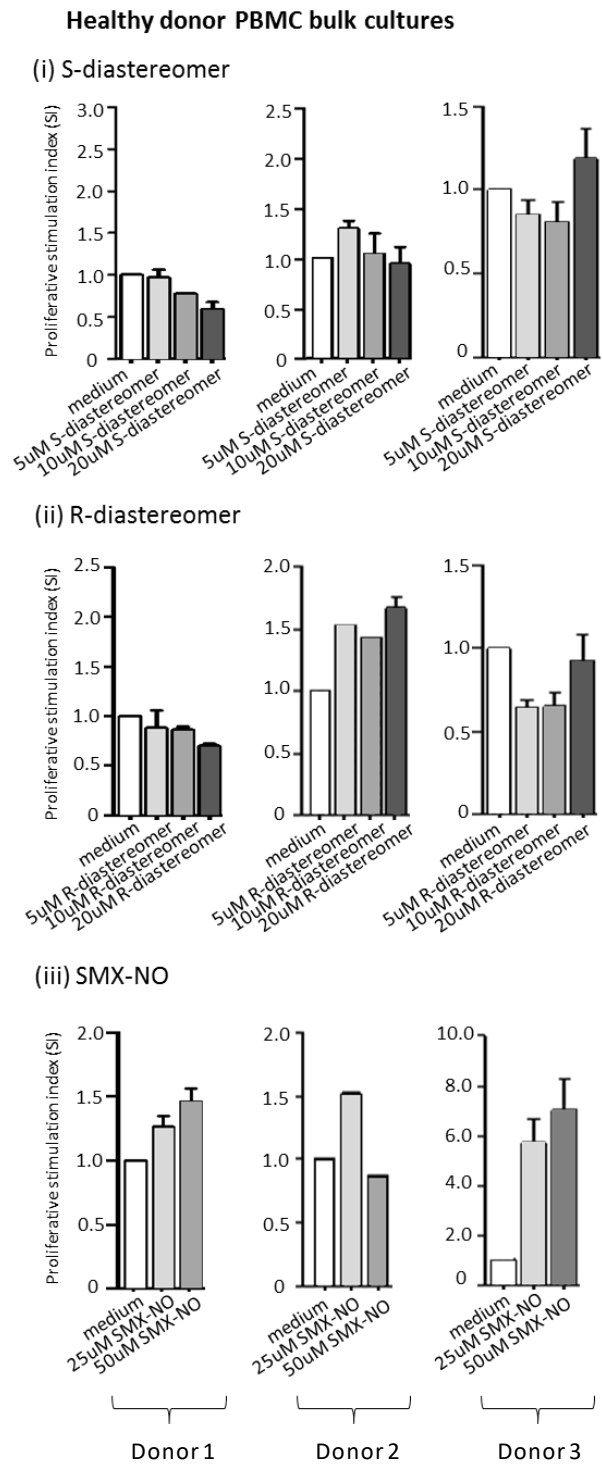
(B) R-diastereomer

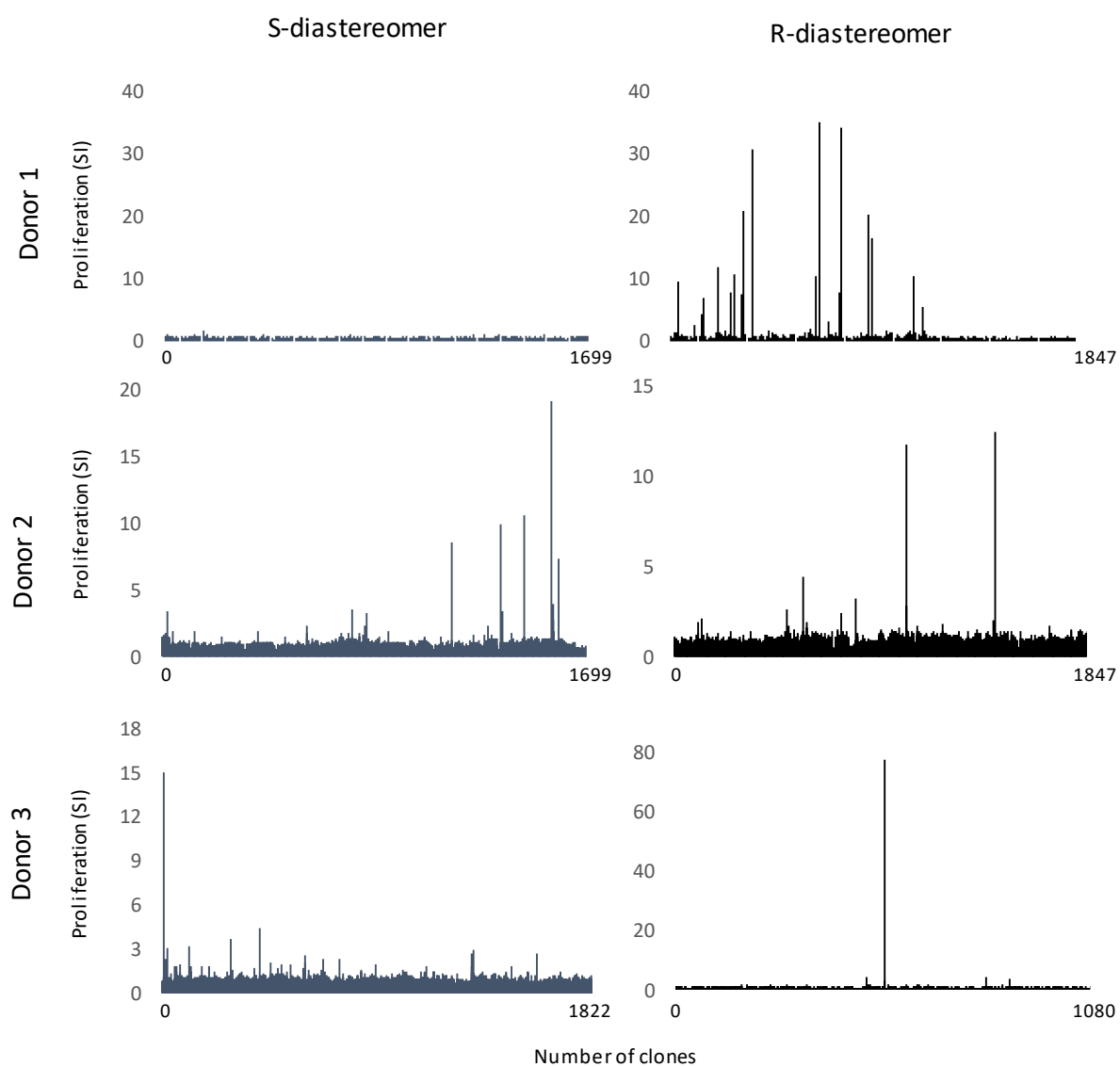


(C) M11 metabolite (VRT-841125)

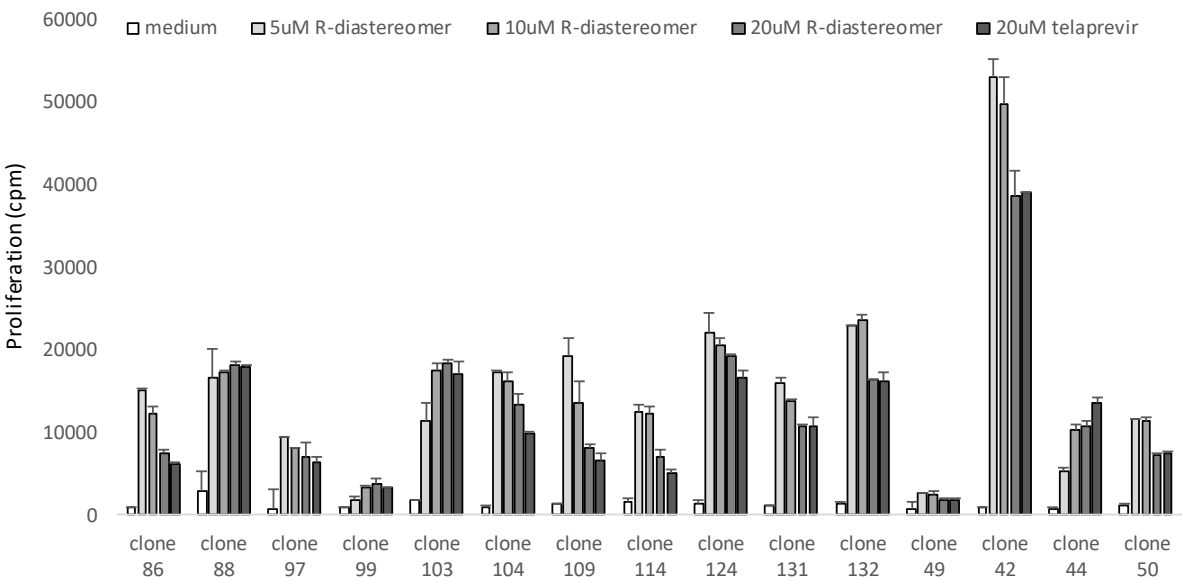




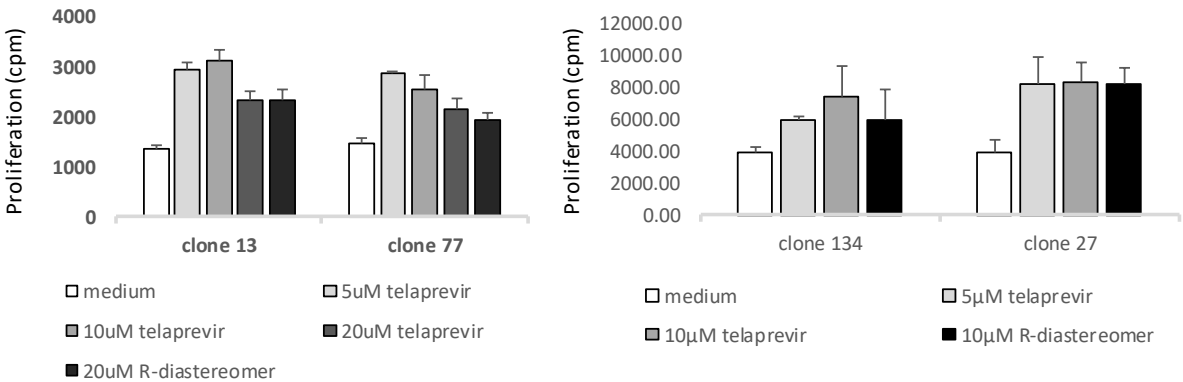


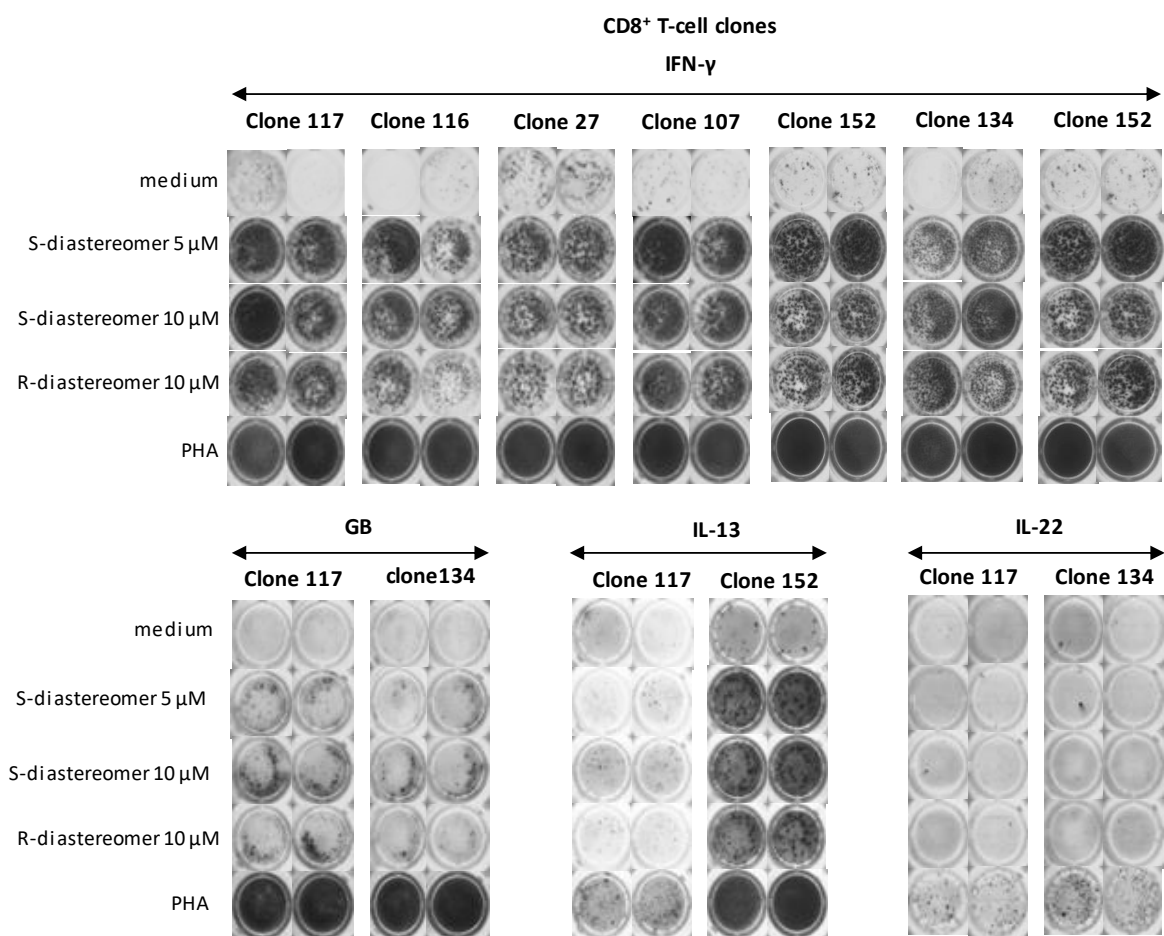


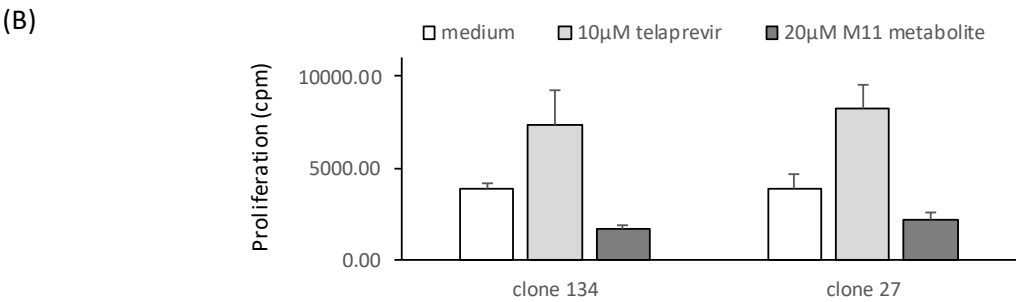
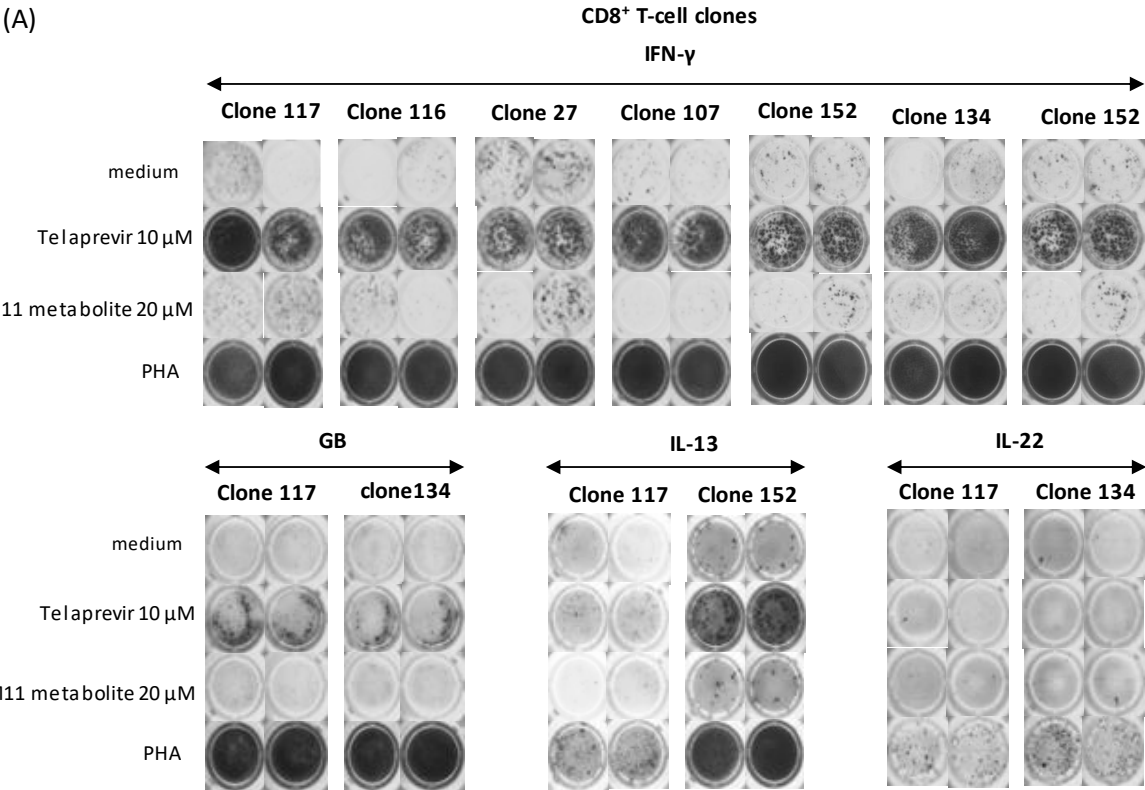
**T-cell clones derived from R-diastereomer cultures**

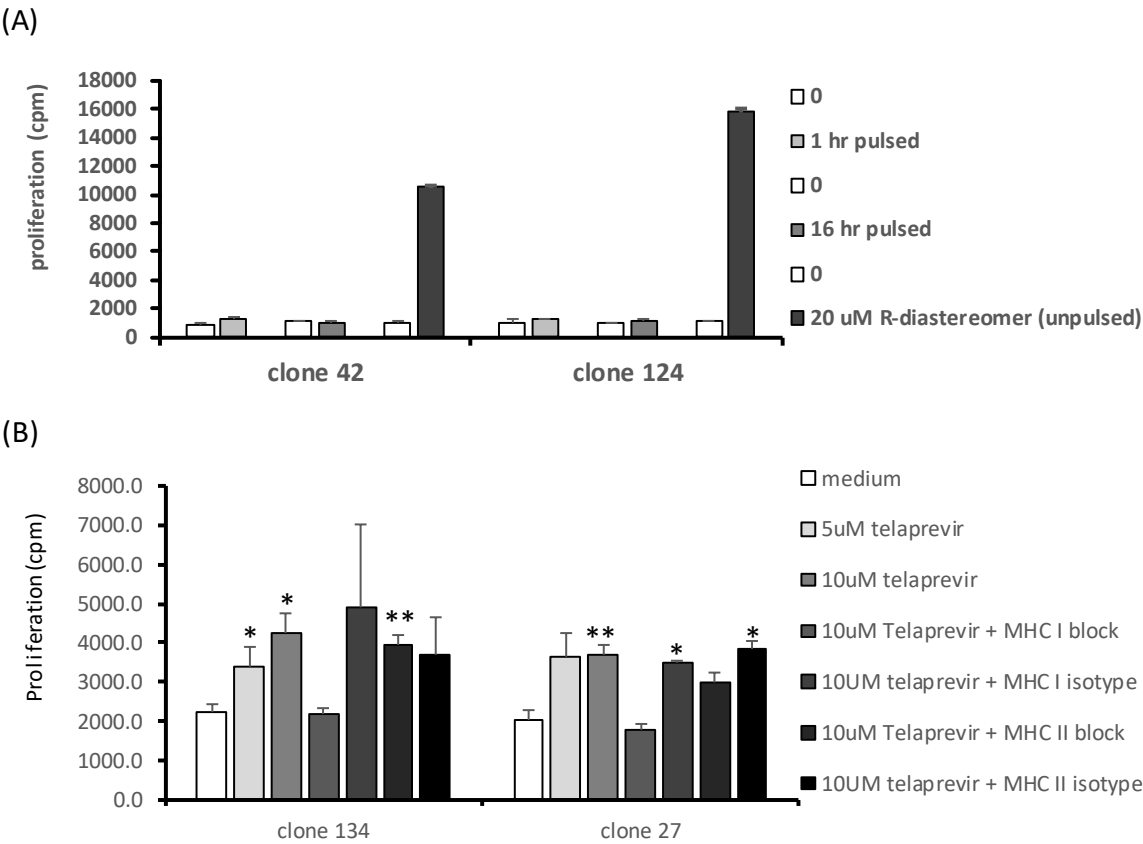


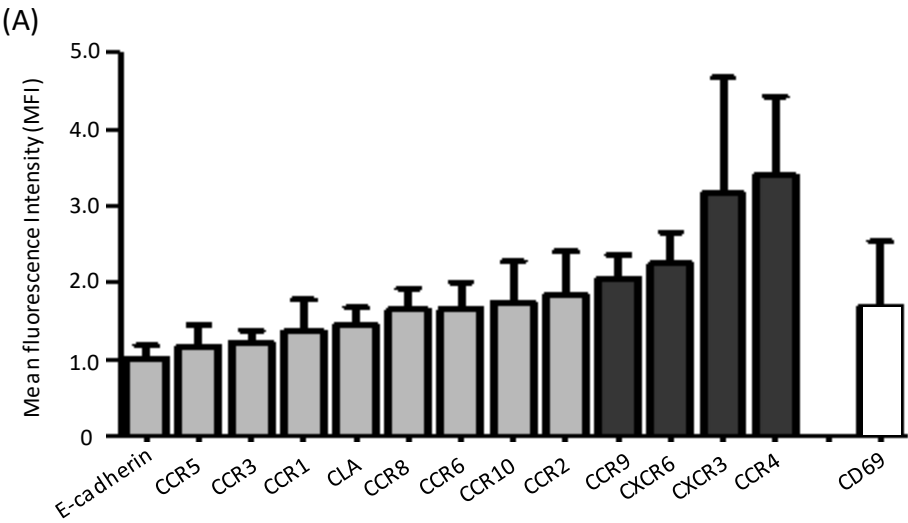
**T-cell clones derived from telaprevir (S-diastereomer) cultures**











CD69	1.7	±	0.8
Chemokines	MFI		
CCR4	3.40	±	1.04
CXCR3	3.16	±	1.52
CXCR6	2.25	±	0.41
CCR9	2.06	±	0.31
CCR2	1.83	±	0.57
CCR10	1.74	±	0.54
CCR6	1.66	±	0.35
CCR8	1.65	±	0.28
CLA	1.44	±	0.23
CCR1	1.36	±	0.42
CCR3	1.22	±	0.15
CCR5	1.17	±	0.28
E CAD	1.01	±	0.17

